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Production of Normal Human Epidermal Keratinocytes for Use in Biochemical Research

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This technical report outlines procedures that have been developed to provide a cost effective and high efficiency quality control quantity of normal human epidermal keratinocyte (NHEK) cells to conduct research into the mechanism of action of chemical warfare (CW) agents and to reduce the dependence on live animal testing with toxic CW agents. Adult NHEK cell lines were grown at different cell densities in a variety of diverse tissue culture vessels. The purpose of this technical report is to provide an overall review of important tissue culture parameters for the optimal production and use of NHEK in toxicological research. These cells have provided valuable information for the design of therapeutic intervention against 2,2'-dichlorodiethyl sulfide (sulfur mustard, HD) induced lesions.

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Introduction

Cultures of normal human epidermal keratinocytes (NHEK) retain many of the characteristics of the tissue from which they are derived and are, therefore, useful as an experimental model for studying biochemical properties (Watt, 1988). The cultivation of human keratinocytes is an appropriate experimental model for a variety of biological, pharmacological and biochemical investigations, and the production of *in vitro* NHEK has been well established. Skin is a major target organ for many alkylating and carcinogenic agents that exist in our environment, and the majority of previous carcinogenicity studies have used animal-derived models (Eckert, 1989). Since many of these chemical threats exhibit species- and tissue-specific metabolism, a human skin tissue derived model would be a distinct advantage. Human epidermal keratinocytes present an appropriate target cell to employ as an *in vitro* system to study epidermal chemically induced cellular injury. Consequently, these cells are a valuable model for evaluating prophylactic and therapeutic compounds. These statements have been corroborated in-house by the observation that basal epidermal cells are the most proliferating cells in the upper layers of the skin and are the major target of alkylation-induced cellular injury (Smith, Sanders, Caulfield, and Gross, 1992).

The purpose of this technical report is to provide an overall review of important tissue culture parameters for the optimal production and use of NHEK in biotoxicological research. This report will focus on standardized methods of culturing, variances of media, batch to batch cell variations, adult versus neonatal cells, and varieties of growth vessels.

Methods

Media

Keratinocyte Growth Medium (KGM) is a sterile filtered medium developed by Clonetics Corporation to feed proliferating NHEK. It is used to rapidly establish or sustain proliferating cells. There are several different keratinocyte growth media formulations: KGM, KBM, KBM-2, and KBM-D (defined Medium). Basic differences among the various formulations are the growth rate and phenotypic appearance of the cells.

Cells

NHEK from Clonetics Corporation are frozen in a cryoprotective cocktail (growth medium, 10% v/v fetal bovine serum, and 10% v/v dimethylsulfoxide). The cells are shipped from Clonetics in a liquid nitrogen Dewar flask. Upon arrival at the laboratory, they are removed from the Dewar and immediately returned to liquid nitrogen storage at -170^0 C.

Culture Vessel

The type of assay being performed determines the style, size, and quantity of culture vessels required by the investigators. Culture vessels are obtained from several sources, such as Corning (N.Y.) and Falcon (Becton Dickinson and Company, New Jersey).

The Corning Corporation uses virgin medical grade polystyrene in their flasks and multiple well plates, which provide optical clarity. Corning also modifies the surface of these vessels using a special process, thereby increasing protein binding and cell attachment (Corning Disposable Multiple Well Plates, TC-CGW-50 805810 leaflet).

The Falcon Corporation has developed a permanent, stable modification of the polystyrene surface of culture vessels (Primaria). This surface contains a mixture of anionic and cationic functional groups, thus improving the attachment of cells. The plates are obtained pre-sterilized by gamma radiation.

Initiation of Growth from Cryovial

The cryovial is removed from the liquid nitrogen freezer, placed in an insulated container with dry ice, and transported to the cell culture laboratory.

A sterile field within the BioChem Gard hood Model BL-6 (Baker Company, Inc. Sanford, Maine) is prepared by wiping down all exposed surfaces with 70 percent ethyl alcohol. Before cryoretrieval, KGM is warmed to 37^0 C in a Precision stainless steel water bath (Model 138). The pre-warmed bottle of KGM is removed from the water bath, dried with a paper towel, and sprayed with 70 percent ethyl alcohol before being placed in the sterile field. Thirty milliliters of warm KGM is added to a sterile 50-milliliter conical centrifuge tube.

The cryovial is removed from the insulated cryo-freezing container (Nalgene) and immediately swirled in the water bath at 37^0 C. The swirling action produces a more even distribution of the temperature throughout the vial and reduces temperature gradients within the suspension. When only a small amount of the frozen cell suspension remains in the cryovial (approximately two minutes at 37^0 C) it is removed from the water bath. Cryopreserved cells are very delicate and must be thawed and returned to culture as quickly as possible with minimal handling. The cryovial is dried with a paper towel, sprayed with 70 percent ethyl alcohol and placed in the BioChem Gard hood. The cap of the cryovial is removed using a 70 percent isopropyl alcohol pad (WEBCOL). The cells are transferred to a sterile 50-milliliter conical tube using a one-milliliter pipette. The cell suspension is gently mixed with a 25-milliliter pipette. Fifteen milliliters of this cell suspension is added to each T-75 flask.

The side of each flask is labeled using a permanent marker, and the markings include cell type, date, passage number and the technician's initials. These flasks are placed on a mechanical agitator (Belly Dancer, Stovall Life Science, Inc.) for approximately five to ten minutes to enhance even distribution. They are placed in a Steri-Cult 200 HEPA filtered infrared (Forma Scientific) CO_2 incubator at 37^0 C, with a 5 percent CO_2 level. The lids to the flasks must be opened at least one quarter of a turn for CO_2 gas exchange if the caps are not vented. After 24 hours, the KGM is replaced with fresh medium to remove the remaining dimethylsulfoxide (DMSO) from the cryoprotective cocktail. Clonetics also recommends changing the growth medium on alternating days in an effort to achieve a 50 percent plating efficiency. These cells are observed daily for growth rates and cell condition. Cultures should have cells containing a regular morphology and clear nongranular cytoplasm (Recovery of Cells From Cryopreservation, TM7030A, Rev. #4, leaflet, 1992).

Subculture Technique

The T-75 flasks are examined microscopically using an Olympic inverted microscope (Model CK2), and confluence of the cell monolayer is estimated. When confluence is approximately 80 percent, the subculture procedures begin. The type of vessels and concentration of cells per vessel are predetermined by the requirements of each research team. Sterile field preparation of the BioChem Gard hood is achieved by wiping down all surfaces with 70 percent ethyl alcohol. The KGM stock bottle is also prepared by wiping down the Bovine Pituitary Extract (BPE) supplement from the medium bottle with 70 percent ethanol. The contents of the BPE vial are aseptically added to the KGM medium bottle, recapped and mixed by gentle swirling (KGM, Instructions For Use, leaflet). The bottle is labeled with the date and the technician's initials.

The reagents are warmed to 37 ^{0}C by incubation in a water bath. The Clonetic EpiPack is thrawed in the Precision water bath. This package contains the following reagents: Hepes buffered saline solution (HBSS) (CC5022), Trypsin/EDTA solution (CC-5012), and Trypsin neutralizing solution (CC-5002) (EpiPack, leaflet). Only the amount of reagents required to perform the process should be warmed since continuous heating shortens the life of the medium.

The T-75 flasks are placed in the hood, 7 milliliters of Hepes buffer saline solution is added to each flask, and flasks are placed on the mechanical agitator and gently swirled for two minutes. The flasks are returned to the sterile field, and the HBSS is removed using a one-milliliter sterile pipette connected to a vacuum line. Seven milliliters of trypsin/EDTA is added to each flask. The flasks are placed in the incubator for seven minutes. After seven minutes, the flasks are monitored microscopically for detached cells, which are rounded and floating. Dislodging the remaining cells is accomplished by lightly rapping the flask against the palm of your hand. Caution should be exercised during this procedure since excessive rapping of the flasks can crack them and produce fine bubbles destroying cells that attach to the bubbles. The trypsin should not be in contact with the cells for longer than ten minutes. After cells are detached from the flask surface, seven milliliters of trypsin neutralizing solution is rapidly added.

The cell suspension is transferred to centrifuge tubes and spun in a Beckman GS-6R centrifuge at (200 x g), 15 ^{0}C for 5 minutes forming a cell pellet. The supernatant is discarded and the remaining cell pellet is resuspended in ten milliliters of KGM. An aliquot of 50 microliters of the cell suspension and 50 microliters of trypan blue is mixed in a small microcentrifuge tube and placed in a Fisher Scientific hemacytometer to determine a cell count. Cells are counted microscopically using a Fisher Differential Counter, Model III. Cells can also be counted electronically using the Coulter Particle Counter (Z-Series).

The cells are subcultured into flasks, plates or inserts. Various assay parameters determine which cell concentrations are placed in the appropriate culture vessels. According to Clonetics, 80 percent of the cells should attach during the first 24-hour period. The cells should be single or in small groups. The appearance of mitotic cells after 24 hours is an indication of active cell growth. To optimize active cell growth the media should be changed on alternate days.

Cell Counting

Cells can be counted manually in a hemacytometer or determined by electronic measurement. The hemacytometer is made from a thickened glass slide that contains a small chamber divided into nine sections. Only four sections are required to accurately count the cells if at least 100 cells are present. A 1:2 dilution is prepared by withdrawing a 50-µl aliquot of cell suspension and adding the same volume of trypan blue. The suspension is thoroughly mixed using an Eppendorf pipette. A cover slip is placed over the hemacytometer chamber. The hemacytometer chamber is filled slowly and steadily, because overfilling, underfilling or injecting bubbles into the chamber will produce an incorrect cell count. Cells are counted in four 1-mm corner squares. Cell count per volume of suspension is determined by the following formula.

$$\frac{\#\ cells\ counted}{\#of\ squares\ counted} X\ dilution\ factor\ X\ 10^4 = cell\ count\ per/ml$$

When possible, cell counts are determined on a Coulter Particle cell counter, series Z. This instrument is calibrated using standard size latex beads and routinely compared with hemacytometer counts.

Results/Problems

Trypsinization Process

The trypsinization process is initiated when confluence reaches 60 to 80 percent and cells are in the rapid growth phase. When 90 percent confluence is reached, mitosis is arrested and reduced cell viability occurs. Cells can peel off surface areas due to the formation of a flexible extracellular matrix and can become more strongly attached to the matrix than to the plastic substrate. The sheet tears or pulls away from the flask because of mechanical stress produced by cellular movement and contractions (Corning Corporation, leaflet TC-CGW-99, March 87). The cells become difficult to remove from the flasks and trypsin must remain on the cells for a longer period. However, extended exposure to trypsin compromises the NHEK membranes and the cells will lift off in sheets. A reliable cell count is impossible under these conditions, and uniform distribution into wells or flasks is futile. Consequently, vessels will contain areas of both high cell concentration and low cell population. If these cells are extremely isolated, they are unable to communicate intercellularly with neighboring cells through the large volume of medium. Without intercellular communication the cells will not proliferate and will die. Cells that are located closely together will reach a high level of confluence rapidly and will begin to differentiate before the remaining areas of the flask reach the required confluence. The areas of high cell concentration grow rapidly but die off quickly, before the remaining surface area can become confluent. Cells in this high-density area will begin to peel off in a layer of the flask surface, and cell death occurs in areas where the seeding density is too low. These vessels are unusable and must be discarded.

Contamination

Incubator

A major source of contamination is the incubator since older style units are difficult to disassemble and clean thoroughly. All shelves, side panels, water pans, gaskets and fan covers of the incubator must be removed. The inside chamber is cleaned with a phenol reagent, rinsed, then sprayed with 70 percent ethanol. The removable pieces are cleaned in the same fashion then autoclaved for 20 minutes at 15 psi. The filter must be replaced. If this process does not eliminate the contamination, the unit can be sterilized using formaldehyde gas by contracted maintenance personnel.

The water pan in an incubator can be another major source of contamination if not maintained correctly and must be cleaned with a disinfectant (i.e., ChlorhexiDerm, Nolvasan) biweekly and periodically autoclaved. Bacteria, molds, and yeasts are the most common contaminants; they grow rapidly and quickly colonize in cell cultures (Ryan, 1994).

The Steri-cult 200 HEPA filtered infrared CO_2 incubator (Forma Scientific) has eliminated several problems. Contamination and desiccation of cultures is reduced because this system uses vertical laminar airflow technology as well as a HEPA filter, which returns contaminant-free air to the chamber and is replaced every five years. The warm air jacket provides a uniform temperature and reduces condensation on the inner door.

The Steri-cult 200 does not require a water pan to humidify the internal environment but contains an internal chamber that performs this function. The chamber holds approximately 3 liters of sterile, distilled water. Absorbent paper towels should be placed on the top of the unit while adding sterile, distilled water. If the water splashes on the front panel, a short can occur in the temperature monitor and the reading will be incorrect. If this problem occurs, Forma Scientific recommends sliding the temperature monitor slightly out of the panel and blowing air into the module to expedite the drying process, and then replacing the module in the panel. The reading should return to normal in approximately 15 minutes.

Several technicians using the same incubator will cause the door to be opened more frequently altering the CO_2 level, temperature and humidity as well as exposing the culture vessels to room air. Temperature fluctuation inside the incubator is evident when heavy condensation is formed on vessels located at the front of the incubator. Slightly cooler temperatures produce slower growth rates, and the vessels are more prone to fungal contaminants from the condensation. The technician's hands inside the chamber can also provide an additional source of contamination. When additional personnel use the same laboratory, it must be cleaned more frequently.

The location of a vessel inside the incubator can also affect the growth rate. Vessels in contact with metal shelves warm up quickly when first placed in the incubator, but the vessels stacked on top will cool off quickly. The vessel in the middle positions have the least amount of temperature fluctuations due to the insulating effects produced by the other vessels. If possible, vessels should not be stacked, but use of empty vessels under the inoculated vessels can eliminate the rapid heating effect. Shelves that are not level cause uneven distribution of cells across the surface of the flasks and especially the wells of plates.

The incubator should be placed in a location of minimal traffic since movement of personnel near the incubator disturbs the room air and increases the amount of particulate matter

such as dust. If the unit is placed too close to the hood, the airflow pattern is disrupted by the negative airflow in the hood. The incubator should not be placed directly on the floor because there is an increased possibility of contaminating the unit when the floors are mopped. Rollers can be purchased to elevate the unit.

Different cell types such as HeLa cells should not be placed in the incubator with NHEK since these cells have the potential for cross contamination.

Water Bath

The water bath should be cleaned on a routine basis by a non-abrasive cleanser and rinsed thoroughly. The bath is dried, sprayed with 70 percent ethanol, wiped with a towel, and then filled with distilled water. Caution should be used when adding a clear bath algaecide since this substance is highly toxic and corrosive, and can destroy the finish in the stainless water bath. The temperature should be checked to ensure the settings are not disturbed during the cleaning process.

Glass Door Refrigerator

Room temperature and humidity fluctuations produce sweating on the glass doors of the refrigerator and must be periodically wiped off to remove this excessive moisture. If this process is not performed, the track in which the glass doors slide fills with water, producing mold and mildew. Before a new shipment of media is placed in the unit, the interior walls and shelves should be cleaned with a non-abrasive cleanser.

Vacuum Line and Waste Container

Before beginning any cell culture procedures the vacuum line is sprayed with 70 percent ethyl alcohol, and the vacuum is allowed to run for several minutes to ensure that the alcohol is pulled through the line. When the four-liter vacuum flask is filled, the vacuum is turned off and the flask is disconnected from the system. Bleach is added to the waste until a color change indicates a pH change. The liquid waste volume, date of disposal and signature of the technician are recorded on the sink log before discarding. The flask is washed with water, rinsed with bleach and returned to the plastic container that holds the flask. Were the flask to crack, the plastic container would prevent the waste from spilling on the floor. When the four-liter flask is in the container, 500 milliliters of Clorox bleach is added to the flask before the vacuum line is reattached. The vacuum is turned on; the line is sprayed and allowed to run for several minutes. Approximately once a week, the line is rinsed with bleach. At the end of each cell culture, the line is rinsed with alcohol.

Used Plastic Ware

Used pipettes are temporarily placed in a plastic container within the operators' reach, near the hood. After the cell culture procedures are finished, the pipettes are immediately removed and placed in the glass disposal box. The plastic container is rinsed, dried and then sprayed with 70 percent ethanol.

Waste (Plastic and Paper)

A large plastic trash bin is located near the operators' reach and is used for discarded items such as empty media bottles, tripack reagent bottles, and plastic/paper wrapping from flasks, plates, and pipettes. When the trash bin is full the plastic liner holding the trash is removed, tied and taken to the dumpster for disposal, and a new liner is placed in the bin. When not in use, the bin is kept away from the working area to reduce the possibility of contamination.

Laboratory

The laboratory should be cleaned weekly. All exposed surface areas such as bench tops, exterior hood, centrifuge casing, and microscopes should be wiped down with 70 percent ethyl alcohol. The floor should be mopped with a disinfectant. The bucket and mop should never be stored in the laboratory since they provide a good source for the production of mildew, mold, and fungus.

Laboratory Personnel

The largest source of contamination in a cell culture laboratory is the operator. The amount of contamination increases as the number of technicians sharing the same work area increases. Technicians learning *in vitro* techniques can have poor aseptic techniques but improve with practice. During that practicing period, however, there is an increase potential for contamination. Before performing any cell culture procedure (including preparing the sterile field within the hood), technicians should wash their hands with antibacterial soap before gloving and put on a clean buttoned laboratory coat. The gloves and laboratory coat should not separate and expose the technicians' wrists. This problem can be resolved by pulling the gloves up over the bottom of the lab coat sleeves. If this is not possible, disposable gauntlets can be worn.

Minimizing the number of technicians using the same area can dramatically reduce contamination. Technicians should never use media and reagents that were prepared by another individual. Each container should be marked with the date it was opened and the initials of the user. These common sense procedures reduce the opportunity for contamination.

Equipment

Plastic Ware

The plastic ware can affect the growth pattern and alter confluence Differences in plastic ware exist between manufacturers as well as between different lot numbers. Problems encountered consist of inconsistent, spotty and uneven cell attachment, which cause growth rates that change gradually or abruptly.

Troubleshooting

The Corning Corporation (leaflet TC-CGW-99) uses a seven-step method to troubleshoot common problems. First, a problem must be well defined and identified. It may be necessary to repeat the problem to locate it. If a complex problem exists, the second step is to divide it into smaller, simpler pieces. Third, look for cause and effect relationships. Use care not to discard the obvious. Fourth, never try to correct a problem by changing everything at once. Changing everything removes the possibility of ever locating the original source of the problem. The fifth step is to use everyone's knowledge and experience to brainstorm and search for possible causes. All changes that have occurred in the laboratory must be identified (cultures, media, etc.). Good recordkeeping is essential to track the problem. The last step is to determine and implement solutions. A well-run cell culture laboratory will use this information to fix the original problem, and minimize or prevent a recurrence of similar problems.

Cells and Reagents

Cell Considerations

Clonetics provides a Certificate of Analysis for each donor. This information allows new lot numbers to be selected based on viability, seeding efficiency, and growth rate. The quantity of available cryovials is also taken into consideration.

Neonatal cells in general will have a faster doubling time. However, most investigators at the MRICD prefer cells from adult donors.

KGM versus Bullet Kits

The KGM formulation has a shelf life of six to eight weeks, and Bovine Pituitary Extract (BPE) must be added to the KGM before it is ready to use.

After BPE is added, the shelf life is approximately two weeks. The KBM formulation has an extended shelf life of eight months. The bullet kit must be added before it can be used. After the addition of the bullet kit, the shelf life is two weeks.

Each new batch of KGM can be slightly different, thus affecting the growth rate. The KGM bullet kit is based on modified MCDB-153. It contains BPE, hormone epidermal growth factor (hegf), insulin, hydrocortisone, Gentamicin, and Amphotericin-B-1000. The KGM-2 bullet kit is based on Clonetics Corp. Media Development Laboratories CCMD-151. It contains Bovine Pituitary Extract, hegf-b, insulin, hydrocortisone, transferrin, epinephrine and Gentamicin, Amphotericin-BGA-1000. KGM-D (Defined Environment) is based on Clonetics Corp. Media Development Laboratories CCMD-150. It contains hEGF, rHu insulin, hydrocortisone, Prostaglandin E2, epinephrine, and Gentamicin, Amphotericin-B-100.

Three different bullet kit formulations of growth media were compared with KGM. In comparing the different media formulations (keeping all other factors constant such as flask type, cell lot number, seeding density and volume of media per culture vessel), it was found that the type of media used not only affects the growth rate, but also alters the phenotypic appearance of the NHEK.

Cells grown in KGM-2 reach 60-80 percent confluence several days earlier than cells grown in KGM. However, these cells had a fibroblastic phenotypic appearance. The cells were long and thin. The NHEK did not have the cobblestone appearance that the investigators expect to find in their cultures. These cells had a flat appearance, and many individual cells were extremely large compared with cells grown in KGM.

Differences among clusters are noticeable between KGM and KGM-2. Individual cells are on the outside edge of the clusters with KGM; however, the edges of the clusters grown in KGM-2 are smooth. When the flasks are 70-80 percent confluent, elongated cells are present. These cells appear to be connecting the clusters.

Bovine Pituitary Extract Supplement

Investigators at the US Army Medical Research Institute of Chemical Defense found long, stringy fibers in the culture vessels, which became more noticeable as the level of confluence increased. Rinsing the vessels several times with Hepes did not remove the fibers. Scientists were reporting the substance as contamination and discarding the culture vessels. Attempts were made to isolate and identify the substance. Several different types of agar plates tested did not show any type of contamination. The flasks were given to the Comparative Pathology Branch to determine the type of contamination. Their findings were also negative. EM photographs were taken of the substance and sent to Clonetics along with suspect flasks containing the fibers. Several different lot numbers of KGM and KGM with bullet kits were tested. It was noted that flasks containing media with the bovine pituitary extract (BPE) additive produced the fibers. Bovine pituitary extract is chemically undefined but is required for growth of the NHEK in culture.

Cells grown in KGM-D did not contain the long, stringy fibers that are produced when bovine pituitary extract is added to complete the media. To eliminate the fibers, defined media was used. Unfortunately, the growth rate of the NHEK in KGM-D was poor. Cells obtained from Clonetics are isolated and grown in KGM before cryofreezing. Changing to KGM-D slows the amount of time required for cell division and changes the appearance of the cells. Unfortunately, the first cell divisions did not occur within the first 24 hours but required 48 to 72 hours before the cells began to divide and form clusters. Cells fed KGM-D did not produce a cobblestone like appearance. These cultures contained large cells and fewer clusters than what was observed when using a more enriched formulation such as KGM. Cells grown in KGM-D develop extremely slowly. Many areas in the flask die quickly, possibly due to the low cell density in those areas.

Batch-to-Batch Variations

Each new lot number of NHEK is produced from a different donor. The seeding efficiency and the cell viability are different for each lot number. The amount of time required for the cells to double is dependent upon factors specific to the individual donor, such as age. These factors must be taken into consideration when determining seeding densities. Each cryovial contains approximately 500,000 cells. Normally, 250,000 cells are placed in a T-75 flask for 7-8 days to reach the 60-80 percent required confluence. However, certain lot numbers respond differently. The same parameters can be obtained with other lot numbers by altering the

seeding density and flasks size. The lot number 2426, currently used by ICD scientists, is adjusted by reducing the seeding density from 250,000 to 6,250 cells per flask. In addition, the surface area of the flask was doubled. This was accomplished by replacing a T-75 flask with a T-150.

Passage Three (P3) Requirements

The requirements that must be obtained for the P3 stage are determined by the individual investigators. The culture vessel, size, type of growth media needed, and the specific lot number required must all be taken into consideration, and alteration of any one of these factors affects the growth rate.

There are slight differences in batch to batch formulations of media that can alter the growth rate. Each group of plastic ware for each investigator should come from one manufacturer as well as from the same lot number. If the same cell suspension is used to seed plastic ware acquired from several different sources, the growth rate and pattern of distribution will not be the same for each culture vessel even though the flask size and cell suspension remains constant. Frequent adjustments are required to compensate for these factors to meet the needs of the investigators.

Lot number 4075; Passage 2; Flask size T-75

Quantity of flasks	Total count	Average per flask	Days
2	11,000,000	5,500,000	7
3	17,850,000	5,950,000	6
3	20,250,000	6,750,000	7
3	14,880,000	4,960,000	7
3	13,150,000	5,325,000	7
2	13,800,000	6,900,000	8
4	21,081,000	5,270,250	7
2	8,900,000	4,450,000	8
2	10,000,000	5,000,000	8
2	8,150,000	4,150,000	7
2	13,440,000	6,720,000	8
2	11,100,000	5,550,000	7
2	8,000,000	4,000,000	7
2	7,000,000	3,500,000	7
2	12,000,000	6,050,000	7

Lot number 2426; Passage 2; Flasks T-150

Quantity of flasks	Total count	Average per flask	Days	Seeding density	Confluence
10	87,500,000	8,750,000	8	50.0K	60-70%
4	52,500,000	13,125,000	8	62.5K	80-90%
7	90,500,000	12,928,571	10	62.5K	70-80%
8	88,970,000	11,121,250	9	50.0K	100%
5	62,100,000	12,420,000	7	83.3K	100%
4	72,600,000	18,150,000	8	125.0K	100%

Lot number 4075; Passage 3

Flask size	Quantity of flasks	Days	Seeding density	Cell count	Count per flask
T-75	3	8	200 K	5,786,000	1.9 X10^6
T-75	3	8	400 K	7,668,000	2.6X10^6
T-150	3	8	400 K	7,950,000	2.67X10^6
T-150	3	8	800 K	13,038,000	4.33X10^6
T-175	8	10	200 K	19,680,000	2.46X10^6
T-175	4	10	300 K	12,900,000	3.22X10^6

Growth Chart

Based on user needs and amount of cells needed to seed flasks

Lot #	Passage	Days	Flask size	Seeding density	KGM	KGM-2	KGM-D
					confluence using different growth media		
4075	P3	5	T-150	300K	10 %	15-20%	> 5%
4075	P3	8	T-150	300K			
4075	P3	8	T-75	150K			
4075	P3	8	T-75	100K			
4075	P3	7	T-150	300K	50%		
4075	P3	7	T-150	400K	80%		
4075	P3	7	T-150	600K	80%		50%
2426	P3	7	6 WELL	50K	100%		
4075	P3	7	6-WELL	100K	100%		
2426	P3	8	T-150	300K	50-60%	60-70%	30-40%
2426	P3	8	T-75	150K	90%	80%	60-70%
4075	P2	8	2-T75		90%		
4021	P2	8	2-T75		10%		
4162	P2	8	2-T75		20%		
4075	P3	10	T-75	100K	100%		

Seeding to Meet Needs

The cryovials containing 500,000 cells per ampoule are normally setup in two T-75 flasks. After the cells have attached to the culture vessels, the KGM is replaced with fresh media to remove the dimethylsulfoxide (DSMO) preservative used during the cryofreezing procedure. This chemical is toxic to the NHEK and should not be left in contact with the cells for more than 24 hours. The medium is changed on alternating days until the flasks have reached 80 percent confluence (approximately seven days).

Passage three NHEK are normally used when the cells reach approximately 60-80 percent confluence. However, some assays required a 100 % confluence level. To meet the requirements of the investigators, seeding density changes are required to produce the requested confluence during the appropriate period. Many experiments require several days to complete, so these culture vessels must reach the correct conditions on a specific date or the experiment cannot take place. These factors must be taken into consideration while making the seeding density adjustments.

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